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The role of early signaling events in plant-insect interactions

Running title: early events in plant-insect interactions

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Abstract.

The response of plants to the stress caused by herbivores involves several different defense mechanisms. These responses begin at the plant cell plasma membrane, where insect herbivores interact physically by causing mechanical damage and chemically by introducing elicitors or triggering plant-derived signaling molecules. The earliest plant responses to herbivore contact are represented by ion flux unbalances generated in the plant cell plasma membrane at the damaged site. Differences in the charge distribution generate plasma transmembrane potential (V_m) variation, the first event that eventually leads to the initiation of signal transduction pathways and gene expression. Calcium signaling and the generation of reactive oxygen and nitrogen species are early events closely related to V_m variations. This review provides an update on recent developments and advances in plant early signaling in response to herbivory, with particular emphasis on the electrophysiological variations of the plasma membrane potential, calcium signaling, cation channel activity, the production of reactive oxygen and nitrogen species, and the formation of a systemically moving signal from wounded tissues. The roles of calcium dependent protein kinases and calcineurin signaling are also discussed.

Key words Biotic stress, Calcium signaling, Plant electrophysiology, Reactive oxygen and nitrogen species, Signal transduction, Transmembrane potential (V_m).

Introduction

Over the course of millions of years of evolution, plants have developed defensive strategies against herbivorous insects that have led to myriad molecular interactions at both the genomic and metabolic levels. The ability for an organism to survive depends on its ability to respond quickly and efficiently to external stimuli and to develop effective and sustainable defenses.

1 These defenses are intended to directly protect the plant with toxic compounds (or using
2 mechanical defense structures such as thorns and glandular trichomes) or to indirectly protect it
3 through molecular interactions that may attract predators or parasitoids of the herbivorous
4 attacker (Wu and Baldwin, 2010; Baldwin, 2010). Plants have evolved means to recognize and
5 respond quickly to herbivory. These means include the perception of molecular patterns and
6 defense effectors (Bos *et al.*, 2010; Bonaventure *et al.*, 2011; Maffei *et al.*, 2012), the elevation
7 of cytosolic calcium ($[Ca^{2+}]_{cyt}$) (Reddy *et al.*, 2011), the depolarization of the plasma
8 transmembrane potential (V_m) (Bricchi *et al.*, 2010), ion efflux/influx (Bricchi *et al.*, 2013),
9 mitogen-activated protein kinase (MAPK) activation and protein phosphorylation (Arimura and
10 Maffei, 2010; Arimura *et al.*, 2011), the activation of NADPH oxidase, and the production of
11 reactive oxygen (ROS) and nitrogen (RNS) species (Miller and Mittler, 2006; Bricchi *et al.*,
12 2010; Arimura *et al.*, 2011; Marino *et al.*, 2012). These cascades lead to a rise in the production
13 of phytohormones jasmonic acid (JA) and salicylic acid (SA) (Zipfel, 2009; Consales *et al.*,
14 2012; Erb *et al.*, 2012; Bricchi *et al.*, 2013), an increase in the production of ethylene (Arimura *et*
15 *al.*, 2009; Onkokesung *et al.*, 2010; Diaz, 2011; Scala *et al.*, 2013), the expression of late defense
16 response genes involved in the emission of volatile organic compounds (VOCs) (Wu and
17 Baldwin, 2010; Baldwin, 2010; Maffei *et al.*, 2011; Karban *et al.*, 2011) and the production of
18 toxic compounds (Karbon, 2010). These events start locally at the feeding site but can spread
19 systemically throughout the plant (Wu and Baldwin, 2009; Maffei, 2010; Bricchi *et al.*, 2012).
20 Although the individual responses that comprise these pathways have been widely catalogued,
21 the connections between them and their interdependence have received little research attention to
22 date.

Plant responses to herbivory have been previously reviewed both prior to gene expression changes (Ebel and Mithöfer, 1998; Garcia-Brugger *et al.*, 2006; Maffei *et al.*, 2007; Zipfel, 2009; Arimura *et al.*, 2011) and after gene expression changes (Howe and Jander, 2008; Mithöfer *et al.*, 2009a; Wu and Baldwin, 2010; Baldwin, 2010; Bonaventure *et al.*, 2011; Bonaventure, 2012; Pearse and Karban, 2013). The aim of this review is to provide an update on recent developments and advances in our understanding of early plant signaling in response to herbivory, with particular emphasis on the electrophysiological variations of the plasma membrane potential, calcium signaling, cation channel activity, reactive oxygen and nitrogen species production and the presence of a systemically moving signal from the wounded tissues.

Defense in preparation for attack: the sensitivity of the plasma membrane and the role of symplastic signaling

The use of electrophysiology in the study of plant cells has witnessed a slow and steady increase for a number of purposes in recent years (Ochatt, 2013). Electrical signals are known to travel along the plant at different rates, carrying different messages (Gurovich and Hermosilla, 2009; Volkov *et al.*, 2010; Oyarce and Gurovich, 2011; Volkov, 2012; Volkov *et al.*, 2013). Vm depolarization is correlated to increases in the cytosolic calcium ion levels, ion channel activity and ROS and RNS bursts. All these events occur seconds to minutes after herbivory and are among the earliest plant defense responses.

The plasma membrane recognizes changes in the environment surrounding the cell, starting a cascade of electric signaling that eventually results in specific responses. Leaf damage by insect herbivores implies the direct delivery of elicitors or the indirect generation of plant cell wall-derived elicitors that may bind specific receptors at the plant plasma membrane. Emerging

evidence indicates that many high-affinity receptors for insect herbivores are located in the plant cell plasma membrane (Maffei *et al.*, 2012). The elicitor-receptor reaction produces variations in the V_m , which is defined as the difference in the electrochemical gradient between the interior and exterior of the plant cell. These variations can lead to either more positive (depolarization) or more negative (hyperpolarization) V_m values, and such events eventually lead to the generation of signaling cascades (Zebelo and Maffei, 2012a). In the *Spodoptera littoralis*-*Phaseolus lunatus* interaction, both direct herbivory and the insect's oral secretions (OS) have been demonstrated to induce a rapid V_m depolarization (Maffei *et al.*, 2007; Bricchi *et al.*, 2010; Bricchi *et al.*, 2012). The same response has been shown in higher plant species like *Arabidopsis thaliana* (Zebelo and Maffei, 2012b; Bricchi *et al.*, 2013) and *Ginkgo biloba* (Mohanta *et al.*, 2012), as well as in lower plant species like the fern *Pteris vittata* (Imbiscuso *et al.*, 2009). Interestingly, a significant V_m depolarization was observed in response to almost every stylet puncture during *Myzus persicae* phloem feeding (Bricchi *et al.*, 2012).

It is known that systemic signaling induced by biotic stressors is transduced by either chemical or electrical signals (Masi *et al.*, 2009; Zimmermann *et al.*, 2009; Zebelo *et al.*, 2012; Zebelo and Maffei, 2012b; Baluska and Mancuso, 2013; Salvador-Recatala *et al.*, 2014; Mousavi *et al.*, 2014).

OS from some insect herbivores contain effectors that overcome antiherbivore defenses. Herbivores possess diverse microbes in their digestive systems and salivary glands that can modify plant-insect interactions. For example, Colorado potato beetle (*Leptinotarsa decemlineata*) larvae exploit bacteria in their OS to suppress the anti-herbivore defenses of tomato plants (*Solanum lycopersicum*) (Chung *et al.*, 2013). Furthermore, applying bacteria isolated from larval OS to wounded plants confirmed that microbial symbionts are responsible

1 for this defense suppression. A further demonstration that salivary components are necessary to
2 trigger plant responses to herbivore larvae has been recently provided. The ablation of the ventral
3 eversible gland (VEG) of *S. littoralis* prompted a significant reduction in the Vm depolarization
4 and significantly reduced both the cytosolic calcium concentration ($[Ca^{2+}]_{cyt}$) and the H_2O_2 burst
5 (Zebelo and Maffei, 2012b). Moreover, VEG-ablated larvae induced a reduced defense-related
6 enzyme expression and a reduced emission of plant volatiles (Zebelo *et al.*, 2014).

7 In general, three mechanisms are recognized for the transmission of electrical signals following
8 herbivory: action potentials (APs), variation potentials (VPs) and system potentials (SPs). The
9 OS of insect herbivores are known to cause both APs and VPs, but it is still unclear whether
10 insect herbivory can cause SPs. SPs have been described as novel electrical long distance signals
11 in plants that are induced by wounding, acting as the forerunners of slower chemical signals
12 (Zimmermann *et al.*, 2009). SPs serve as back up APs and VPs and can remain overlapped with
13 APs and VPs in some instances. Although SPs have been demonstrated only in mechanically
14 damaged tissues, it is difficult to exclude the occurrences of SPs following herbivore feeding
15 (Zebelo and Maffei, 2012a).

16 APs comprise a generic long distance signaling system that may act to potentiate a host response
17 to subsequent signals delivered through alternative long distance information packages. An AP is
18 a momentary change in the electrical potential of plant cells that sense stimuli from
19 environmental stressors, eventually leading to intercellular and intracellular communication. A
20 number of substances strongly depolarize the plasma membrane and thus presumably activate
21 voltage-gated ion channels. Although in principle it is possible that (anion) channels are directly
22 activated by depolarization, the temporal sequence of the ion flux kinetics of barley leaves shows
23 that Ca^{2+} is lost from the apoplast well before the apoplastic anion concentration (measured as

1 Cl⁻) starts to increase (Felle and Zimmermann, 2007). Therefore, channel activity is involved in
2 APs. The more the channels are activated, the more rapid the depolarization will be, eventually
3 leading to an accelerated depolarization that is measured as a membrane potential ‘break-
4 through’ typical of an AP. APs generated by herbivory propagate as a fast electrical signals that
5 travel through the entire plant from the point of origin of the perceived input at a speed up to 40
6 cm sec⁻¹ (Volkov, 2012). Zebelo and co-workers demonstrated that herbivore-induced plant
7 volatiles (HIPVs) trigger APs and VPs on nearby receiver plants (Zebelo *et al.*, 2012).

8 It is generally accepted that wounded leaves communicate their damaged status to other leaves
9 through a long-distance process. Using non-invasive electrodes, the surface potential changes in
10 *A. thaliana* were mapped after leaf wounding, and it was found that membrane depolarization is
11 correlated with JA signaling domains in undamaged leaves. Furthermore, of the 34 screened
12 membrane protein mutant lines, the mutations in several clade 3 *GLUTAMATE RECEPTOR-*
13 *LIKE* genes (GLRs 3.2, 3.3 and 3.6) attenuated wound-induced surface potential changes,
14 showing a reduced JA-response gene expression in leaves distal to wounds in a *glr3.3 glr3.6*
15 double mutant (Mousavi *et al.*, 2013). These results open new avenues for research in organ-to-
16 organ wound signaling, demonstrating the existence of plant genes with functions related to
17 those important for synaptic activity in animals. An open question remains: how are electrical
18 signals propagated through the plant body? While animals have a nervous system that is
19 specialized in the conduction of electrical signals, nothing similar is present in plants. Central to
20 the success of these defenses is the need for local and systemic communication between cells.
21 For plant cells, which are surrounded by cell walls, symplastic continuity is achieved through the
22 presence of plasmodesmata (PD). These plasma-membrane-lined channels, bridging the cell
23 wall, provide symplastic continuity and provide soluble and membrane environments for the

1 passage of small and large molecules and the potential for electrical conduction (Maule *et al.*,
2 2011). PD-located proteins (PDLs) are type-I membrane proteins with receptor-like properties,
3 although the nature of their potential ligands is not known (Amari *et al.*, 2010). Using
4 *Arabidopsis* plants mutated for *pdlp* genes, Bricchi and co-workers (2013) not only implicated
5 PDs directly for their role in the defense against herbivory but also showed that some molecular
6 responses to herbivory can be genetically distinguished from each other and from the overall
7 defense response. However, although PDs have been correlated with gap junctions, no synaptic
8 mechanisms of molecules are present to justify what has been demonstrated in the animal
9 nervous system. Little is known about the electrophysiological responses of phloem sieve
10 elements in wounding, and whether natural damaging stimuli induce propagating electrical
11 signals in these tissues. Very recently, the use of living aphids and the direct current (DC)
12 version of the electrical penetration graph (EPG) were used to detect changes in the membrane
13 potential of *Arabidopsis* sieve elements (SEs) during caterpillar wounding. Feeding wounds in
14 the lamina induced rapid depolarization waves in the affected leaf, increasing to the maximum
15 amplitude (c. 60 mV) within 2 s. Major damage to the midvein induced fast and slow
16 depolarization waves in unwounded neighbor leaves but only slow depolarization waves in non-
17 neighboring leaves. The slow depolarization waves rose to a maximum amplitude (c. 30 mV)
18 within 14 s. The distal electrical signals elicited by caterpillar cutting are indistinguishable from
19 those elicited by a purely mechanical stimulus, demonstrating that the mechanical aspect of
20 insect chewing is sufficient to induce the full electrophysiological response recorded in the
21 phloem sieve elements of unwounded leaves (Salvador-Recatala *et al.*, 2014). Moreover, no
22 remote electrical signals were recorded from the sieve elements of *glr3.3a glr3.6a* plants,
23 indicating that GLR3.3 and/or GLR3.6 are necessary for the production of both the slow and fast

(action potential-like) remote depolarization waves. Therefore, it is conceivable that the depolarization waves produced by the sieve elements in response to remote wounding contributed to the global wound-activated surface potentials (Salvador-Recatala *et al.*, 2014).

Genetically encoded voltage-sensitive fluorescent proteins (VSFPs) are being used in neurobiology as non-invasive tools to study synchronous electrical activities in specific groups of nerve cells. This "light-based electrophysiology" has been recently adapted for use in plant systems. The production of transgenic plants engineered to express different versions of VSFPs that are targeted to the plasma membrane and internal membranes of root cells has allowed the recording of concurrent changes in the plasma membrane potential in populations of cells and at multiple membrane systems within single cells in response to various stimuli in living plants. Such coordinated electrical changes may globally orchestrate cell behavior to elicit successful reactions of the entire root to varying and unpredictable environments (Matzke and Matzke, 2013).

Another interesting connection to electrical signaling could be the recent discovery that plants are able to discriminate between the vibrations caused by chewing and those caused by wind or insect song. A vibration-signaling pathway would complement the known signaling pathways that rely on volatile, electrical, or phloem-borne signals. It has been suggested that vibrations may represent a new long distance signaling mechanism in plant-insect interactions that might contribute to the systemic induction of chemical defenses (Appel and Coccoft, 2014). The mechanisms used by plants to detect and respond to mechanical vibration have received experimental attention from several groups (Gagliano *et al.*, 2012; Monshausen and Haswell, 2013). Mechanoreception is thought to begin with the triggering of mechanosensors in the cell wall and/or plasma membrane, causing fluxes of calcium, ROS, and H⁺. These trigger

1 downstream responses involving many plant hormones and the rapid expression of genes that
2 respond early to many plant stresses (Appel and Cocroft, 2014). Vm changes that occur
3 following herbivory have also been associated with the same signaling events.

4 In the next section, we describe recent findings in early calcium signaling and ion concentration
5 variations upon herbivory following Vm depolarization.

6 **Calcium and other ions act as second messengers in plant-insect interactions**

7 The candidate ion species responsible for Vm variations in plant cells following herbivory are
8 calcium (Ca^{2+}), protons (H^+), potassium (K^+) and chlorine (Cl^-). Herbivore feeding causes a
9 dramatic Ca^{2+} cytosolic ion influx limited to a few cell layers lining the wounded zone (Maffei *et*
10 *al.*, 2004; Howe and Jander, 2008). This response is limited to herbivory or biotrophic activity;
11 neither single nor repeated mechanical wounding induces such significant changes in the
12 cytosolic Ca^{2+} ion influx (Bricchi *et al.*, 2010). The fact that single or repeated mechanical
13 wounding alone is not sufficient to elicit significant $[\text{Ca}^{2+}]_{\text{cyt}}$ variations points to oral factors [or
14 herbivore-associated elicitors (Bonaventure *et al.*, 2011)] as triggers for a $[\text{Ca}^{2+}]_{\text{cyt}}$ burst. Insect
15 feeding and isolated insect-derived elicitors are known to cause changes in the Ca^{2+} homeostasis
16 resulting from the tight regulation of protein channels and transporters located in the plasma
17 membrane and organelle membranes (Jammes *et al.*, 2011) and Ca^{2+} sensors (Arimura and
18 Maffei, 2010; Kudla *et al.*, 2010; Batistic and Kudla, 2012). These events have been associated
19 with Vm depolarization (Bricchi *et al.*, 2013). In plants, $[\text{Ca}^{2+}]_{\text{cyt}}$ is maintained in the nM range
20 (100–200 nM), whereas in many organelles and in the apoplast, $[\text{Ca}^{2+}]$ reaches the mM range
21 (Dodd *et al.*, 2010). The dynamics of spatial and temporal Ca^{2+} changes in the cytosol and/or in
22 other compartments of the plant cell are now accepted to generate “calcium signatures”, which

might be responsible for the initiation of specific downstream events that could eventually lead to the appropriate responses (Mithöfer *et al.*, 2009b; Batistic and Kudla, 2012; Short *et al.*, 2012).

Several techniques have been used and developed to localize, measure and monitor $[Ca^{2+}]_{cyt}$ variations. A large number of fluorescent Ca^{2+} indicators are available for studying Ca^{2+} in plant cells (Mithöfer *et al.*, 2009b). The loading of Ca^{2+} -sensitive fluorescent probes into plant cells is an essential step in measuring the activities of cytoplasmic free Ca^{2+} ions with a fluorescent imaging technique. However, barriers to the loading of the test compounds or the Ca^{2+} -sensitive fluorescent dyes could be represented by the low permeability of the cell wall and by a massive cuticle. This would allow the penetration of only a limited number of cell layers, most likely near the infection zone. In addition to bio-luminescent techniques using aequorin, two fluorescent Ca^{2+} indicators have been recently used to successfully demonstrate the induction of Ca^{2+} signatures upon herbivory: Fluo-3 AM (Kanchiswamy *et al.*, 2010) and Calcium Orange™ (Bricchi *et al.*, 2010; Mohanta *et al.*, 2012; Bricchi *et al.*, 2013). Despite their proven efficacy, these two indicators do not allow for a precise quantification of $[Ca^{2+}]_{cyt}$ variations. Another way to fine-tune the Ca^{2+} variations is by using the Yellow Cameleon (YC) Ca^{2+} -sensor (Russell, 2011). Recently, Maffei and co-workers (2014) used a Cameleon YC3.6 reporter protein expressed in *Arabidopsis thaliana* to quantify $[Ca^{2+}]_{cyt}$ variations upon mechanical damage (MD) to leaves, herbivory by 3rd and 5th instar larvae of *Spodoptera littoralis* and *S. littoralis* OS applied to MD. YC3.6 allowed for a clear distinction between MD and herbivory and quantitatively discriminated calcium responses between the two larvae instars (Verrillo *et al.*, 2014). The GFP probes in particular can be targeted to well-defined subcellular locales, enabling high-resolution mapping of these signals within the cell (Swanson *et al.*, 2011).

1 The development of various Ca^{2+} probes from the past six decades, the improvements that have
2 been developed in this field, the limitations of each probe and important points to consider while
3 planning ideal Ca^{2+} imaging experiments in plant science are all topics that have been recently
4 reviewed (Kanchiswamy *et al.*, 2014).

5 *Calcium sensors*

6 Calcium sensors play a major role in calcium signaling upon herbivory. In the standard model,
7 Ca^{2+} -sensor proteins, such as CaM (calmodulin), detect Ca^{2+} signals and subsequently regulate
8 downstream targets to advance the signal transduction cascade (Du *et al.*, 2011). In *Arabidopsis*,
9 7 genes encode the 4 CaM isoforms (*CaM1/4;CaM2/3/5;CaM6;CaM7*), which differ only in one
10 to five amino acid residues (Batistic and Kudla, 2012). *Arabidopsis SIGNAL RESPONSIVE1*
11 (*AtSR1* or *CAMTA3*) encodes a calmodulin (CaM)-binding transcription factor involved in the
12 mediation of biotic stress responses (Galon *et al.*, 2008). AtSR1 is an important component of
13 plant resistance to insect herbivory as well as one of only three described proteins involved in
14 Ca^{2+} /CaM-dependent signaling to function in the regulation of glucosinolate metabolism,
15 providing a novel avenue for future investigations into plant–insect interactions (Laluk *et al.*,
16 2012). Ca^{2+} /CaM-binding is also critical for the AtSR1-mediated herbivore-induced wound
17 response. Interestingly, *atsr1* mutant plants are more susceptible to herbivore attack than wild-
18 type plants. The complementation of *atsr1* mutant plants by overexpressing the wild-type AtSR1
19 protein can effectively restore plant resistance to herbivore attack. However, when mutants of
20 AtSR1 with impaired CaM-binding ability were overexpressed in *atsr1* mutant plants, plant
21 resistance to herbivore attack was not restored, suggesting a key role for Ca^{2+} /CaM-binding in
22 wound signaling (Qiu *et al.*, 2012).

1 In addition to CaM, plants possess many CaM-like (CML) proteins (50 in *Arabidopsis*) that are
2 predicted to function as Ca^{2+} sensors, but which remain largely uncharacterized (Vadassery *et*
3 *al.*, 2012b). Nevertheless, it is known that most CMLs are cytoplasmic proteins and that some
4 CMLs undergo lipid modifications resulting in membrane binding (Batistic and Kudla, 2012).
5 Among the CMLs, two are particularly involved in the plant immune and biotic response:
6 CML43 and CML42.

7 CML43 displays characteristics typical of Ca^{2+} sensors, and its GUS reporter activity strongly
8 increased when *Arabidopsis*-transformed plants were exposed to the defense compound SA
9 (salicylic acid). Therefore, CML43 functions as a Ca^{2+} sensor in the plant immune response as
10 well (Bender *et al.*, 2014). The perception of microbe-associated molecular patterns (MAMPs) is
11 closely connected to plant responses to insect herbivory. MAMPs typically induce a transient
12 Ca^{2+} burst, resulting in a rapid (within seconds) increase in the free cytosolic Ca^{2+} , which
13 subsequently (within minutes) declines to steady-state Ca^{2+} levels (Frei dit Frey *et al.*, 2012).

14 Plant gene expression induced by OS revealed the up-regulation of a gene encoding the
15 calmodulin-like protein CML42, which negatively regulates plant defense. CML42 is localized
16 to the cytosol and nucleus. Its up-regulation is negatively regulated by the JA receptor
17 Coronatine Insensitive1 (COI1), as a loss of functional COI1 results in prolonged CML42
18 activation. CML42 thus acts as a negative regulator of plant defenses by decreasing COI1-
19 mediated JA sensitivity and the expression of JA-responsive genes, independent of herbivory-
20 induced JA biosynthesis. Furthermore, the results indicate that CML42 acts as a crucial signaling
21 component connecting Ca^{2+} and JA signaling (Vadassery *et al.*, 2012a). CML42 is also involved
22 in abiotic stress responses, as kaempferol glycosides were down regulated in *cml42* and impaired
23 in ultraviolet B resistance. Under drought stress conditions, the level of abscisic acid

1 accumulation was higher in *cml42* plants. Thus, CML42 might serve as a Ca^{2+} sensor, with
2 multiple functions in insect herbivory defense and abiotic stress responses. A porin-like protein
3 (PLP), most likely of bacterial origin, was identified in the collected OS of *S. littoralis* larvae.
4 PLP exhibits channel-forming activity and up-regulates CML42 in *Arabidopsis*; however, it is
5 not sufficient to elevate in vivo $[\text{Ca}^{2+}]_{\text{cyt}}$. Because membrane channel formation is a widespread
6 phenomenon in plant-insect interactions, this PLP might represent an example for microbial
7 compounds from the insect gut, which are initially involved in plant-insect interactions (Guo *et*
8 *al.*, 2013).

9 In addition to calmodulin, plants have two other main families of Ca^{2+} sensors: calcineurin B-
10 like (CBLs) and calcium dependent protein kinases (CPKs). After CBL proteins sense Ca^{2+}
11 signatures, these proteins interact selectively with CBL-interacting protein kinases (CIPKs),
12 thereby forming the CBL/CIPK complexes involved in decoding calcium signals (Zhu *et al.*,
13 2013; Kim, 2013). The CBL-CIPK system shows variety, specificity, and complexity in response
14 to different stresses, and the CBL-CIPK signaling pathway is regulated by complex mechanisms
15 in plant cells involving crosstalk with other signaling pathways (Yu *et al.*, 2014). In vivo and in
16 vitro data have indicated that the kinase CIPK26, upon interaction with the calcium sensors
17 CBL1 or CBL9, enhances the activity of the NADPH oxidase RBOHF via phosphorylation
18 (Drerup *et al.*, 2013; Kimura *et al.*, 2013). Moreover, CBL/CIPK may control the activity of a K^{+}
19 channel from the Shaker family, VvK1.2 in grapevine (Cuellar *et al.*, 2013), whereas protein
20 kinase CIPK9 interacts with the calcium sensor CBL3 and plays crucial roles in K^{+} homeostasis
21 in *Arabidopsis* (Liu *et al.*, 2013). Both ROS and K^{+} are involved in plant responses to herbivory
22 (see below). In tea (*Camelia sinensis*) leaves exposed to a mild infestation of green leafhopper
23 (*Empoasca vitis*), a subtractive cDNA library was constructed using the suppression subtractive

1 hybridization strategy. Genes involved in the CBL–CIPK pathway were up-regulated by
2 herbivory. The expression of a CBL-interacting protein kinase 19, *Cs-Ev9*, significantly
3 increased in the Fuzao2 tea cultivar infested by the tea leafhopper (by approximately 18 times
4 with respect to the controls), providing the first evidence for the involvement of the CBL–CIPK
5 pathway in response to herbivory (Yang *et al.*, 2011).

6 CPKs are multifunctional proteins with Ca^{2+} binding and signaling capabilities in single proteins
7 to directly translate Ca^{2+} signals into phosphorylation events (Tena *et al.*, 2011; Boudsocq and
8 Sheen, 2013; Valmonte *et al.*, 2014). Recent studies have shown that CPKs play a significant
9 role in herbivore-elicited signaling cascades. Upon herbivory, the plant responds by activating
10 Ca^{2+} signatures and corresponding CPKs such as CPK3 and CPK13 in *Arabidopsis*. These CPKs
11 transcriptionally regulate the plant defensin gene (*PDF1.2*) independent of phytohormone
12 signaling cascades in response to *S. littoralis* insect damage (Arimura and Maffei, 2010;
13 Kanchiswamy *et al.*, 2010). This regulation occurs through the phosphorylation of the HSFB2A
14 transcription factor, which positively regulates the expression of *PDF1.2* independent of
15 ethylene, JA and abscisic acid. CPK3 also induces the negative feedback regulation of herbivore-
16 induced Ca^{2+} signals, indicating that CPKs can play redundant and specific roles in plant defense
17 (Kanchiswamy *et al.*, 2010).

18 *Ca²⁺-ATPases*

19 Herbivory induces extracellular stimuli that trigger increases in the cytosolic calcium levels,
20 which are detrimental to plants. To cope with such stresses, plants need to develop efficient
21 efflux mechanisms to maintain ionic homeostasis. The Ca^{2+} -ATPases are members of the P-type
22 ATPase superfamily, which performs many fundamental processes in organisms by actively

transporting ions across cellular membranes (Bose *et al.*, 2011). Moreover, ATP-dependent P-type calcium ATPases can modulate the biotic stress response by activating the components of signaling pathways. The plasma membrane and endomembrane-bound Ca^{2+} channels regulate cytoplasmic $[\text{Ca}^{2+}]$ levels, promoting Ca^{2+} homeostasis by mediating the influx and efflux of Ca^{2+} . Furthermore, the type IIB Ca^{2+} -ATPases have a CAM-binding domain, which, upon binding with Ca^{2+} , forms a complex ($\text{Ca}^{2+}/\text{CaM}$) and initiates many signal networks (Huda *et al.*, 2013). Upon herbivory, the initial Ca^{2+} burst is followed by a consistent decrease in the cytosolic $[\text{Ca}^{2+}]$, which implies the involvement of a Ca^{2+} efflux-mediated increased Ca^{2+} -ATPase activity (Maffei *et al.*, 2007).

Potassium channels

Recently, the question on whether Vm depolarization was caused by a calcium-signaling pathway was addressed. An *Arabidopsis* line (*pdko3*) with a mutation in the genes encoding plasmodesmal proteins was found to be defective in some of the typical plant responses to herbivory. Following herbivory and the release of the *S. littoralis* OS containing a putative β -galactofuranose polysaccharide, the mutant line, unlike WT plants, shows an almost complete loss of Vm depolarization. However, both the *pdko3* and wild type (WT) plants show increased accumulation of cytosolic Ca^{2+} . Thus, mutations in genes for plasmodesmal proteins have provided valuable genetic tools for the dissection of the complex spectrum of responses to herbivory and have shown us that the responses to herbivory imply a Ca^{2+} -independent Vm depolarization (Bricchi *et al.*, 2013). To understand the cause of Vm depolarization, the K^{+} channel activity was measured by confocal imaging when *Arabidopsis* plants were damaged by *S. littoralis* feeding or OS introduced to mechanically damaged leaves. Potassium represents the major osmotically active cation in plant cells and is known to be fundamental for plant functions

1 such as the control of membrane potential (Geiger *et al.*, 2009). Upon herbivory, the increased
2 concentration of cytosolic Ca^{2+} triggers the opening of inward rectifier K^+ channels, which are
3 mainly responsible for the herbivore-induced V_m depolarization. In the *pdko3 Arabidopsis*
4 mutant, the connection between increased calcium and K^+ channel activation is broken, leading
5 to significantly decreased V_m depolarization. However, the lower absolute levels of cytosolic
6 calcium in the *pdko3* leaves open the possibility of a threshold effect in K^+ channel activation
7 (Bricchi *et al.*, 2013).

8 Elicitor-dependent accumulation of second messengers such as Ca^{2+} and reactive
9 oxygen/nitrogen species are central to many signaling and regulation processes in plants.
10 However, the mechanisms that govern the reciprocal interrelation of Ca^{2+} , ROS and RNS
11 signaling are only beginning to emerge. NADPH oxidases of the respiratory burst oxidase
12 homolog (RBOH) family are critical components contributing to the generation of ROS whereas
13 Calcineurin B-like (CBL) Ca^{2+} sensor proteins together with their interacting kinases (CIPKs)
14 have been shown to function in many Ca^{2+} signaling processes, including in the control of K^+
15 channels. Figure 1 shows the role of Ca^{2+} signaling pathways following herbivory.

16 **The second line of defense uses oxidative weapons: ROS and RNS**

17 The unraveling of the mechanisms connected to changes in the V_m , $[\text{Ca}^{2+}]_{\text{cyt}}$, ROS and RNS
18 production upon insect perception will undoubtedly shed critical light on the mechanisms used
19 by plants to sense insect attacks (Bonaventure, 2012). ROS and RNS constitute key features
20 underpinning the dynamic nature of cell signaling systems in plants. Despite their importance in
21 many aspects of cell biology, our understanding of oxidative and especially of nitrosative
22 signaling and their regulation remains poor (Molassiotis and Fotopoulos, 2011).

The role of ROS

Emerging evidence has begun to implicate ROS as pivotal redox-based signaling molecules in the plant defense response (Skelly and Loake, 2013). The modification of cysteines in key regulatory proteins by changes in redox homeostasis is appearing as a central responsive molecular switch that controls the function of a variety of regulators of plant defense responses (Yun *et al.*, 2012). ROS such as H₂O₂ can also activate lipoxygenases to initiate the biosynthesis of oxylipins such as JA (Porta and Rocha-Sosa, 2002); indeed, JA treatment alone produces a H₂O₂ burst (Ozawa *et al.*, 2009). Mechanically damaged Lima bean leaves react rapidly and dramatically to H₂O₂ by inducing a strong V_m depolarization. However, leaves wounded by *S. littoralis* already show a reduced starting V_m, with the consequence of dramatically lower or even no responsiveness to H₂O₂ application (Mithöfer *et al.*, 2009a). Thus, the calcium-induced potassium-dependent depolarization of the V_m following herbivory is linked to a reduction in the downstream responses of the attacked leaves to signaling molecules such as H₂O₂ (Bricchi *et al.*, 2010).

The production of ROS such as H₂O₂ depends on the activity of oxidases. Plant NADPH oxidases, also known as respiratory burst oxidase homologues (RBOHs), are the most thoroughly studied enzymatic ROS-generating systems. Our understanding of their involvement in various plant processes has increased considerably in recent years (Marino *et al.*, 2012). The ability of RBOHs to integrate calcium signaling and protein phosphorylation with ROS production, coupled with genetic studies demonstrating their involvement in many different biological processes in cells, places RBOHs at the center of the ROS network of cells and demonstrates their important function in plants (Suzuki *et al.*, 2011). In *Arabidopsis*, a Ca²⁺-signaling network regulates the formation of ROS. After interaction with the calcium sensors CBL1 or CBL9, the

1 kinase CIPK26 enhances the activity of the NADPH oxidase RBOH via phosphorylation. The
2 CBL-interacting protein kinase CIPK26 specifically interacts with the N-terminal domain of
3 RBOH in yeast two-hybrid analyses and with the full-length RBOH protein in plant cells. In
4 addition, CIPK26 phosphorylates RBOH in vitro, and the co-expression of either CBL1 or CBL9
5 with CIPK26 strongly enhances ROS production by RBOH in HEK293T cells. Together, these
6 findings identify a direct connection between CBL-CIPK-mediated Ca^{2+} signaling and ROS
7 signaling in plants and provide evidence for the synergistic activation of the NADPH oxidase
8 RBOH by direct Ca^{2+} -binding to its EF-hands and Ca^{2+} -induced phosphorylation by
9 CBL1/9CIPK26 complexes (Drerup *et al.*, 2013). Moreover, the direct binding of CIPK26 to
10 RBOH negatively modulates ROS production and plays a role in the regulation of ROS signaling
11 in plants (Kimura *et al.*, 2013). In *Arabidopsis*, extracellular ATP causes the production of ROS
12 in intact roots, with the plasma membrane NADPH oxidase AtRBOHC being the major
13 contributor. This resulted in the stimulation of plasma membrane Ca^{2+} -permeable channels,
14 which contribute to the elevation of cytosolic free Ca^{2+} . The disruption of this pathway in the
15 *AtRbohC* mutant impaired the extracellular ATP-induced increase in ROS, the activation of Ca^{2+}
16 channels, and the transcription of the *MAP kinase3* gene that is known to be involved in stress
17 responses, showing that higher plants evolved a mechanism to transduce the ATP signal at the
18 plasma membrane (Demidchik *et al.*, 2009). In the model plant *Nicotiana attenuata*, transcripts
19 of the respiratory burst NADPH oxidase homolog NaRBOHD are rapidly and transiently induced
20 by wounding and are amplified when *Manduca sexta* OS are added to the wounds. The *M. sexta*
21 OS contain fatty-acid-amino-acid-conjugates (FACs), which are responsible for the increase in
22 NaRBOHD transcripts. Silencing NaRBOHD significantly reduced the ROS levels after OS
23 elicitation, without influencing the expression of the pivotal hormones that regulate plant

1 resistance to herbivores. In addition, NaRBOHD-silenced plants were more vulnerable to insect
2 herbivores, especially to the larvae of the generalist *S. littoralis*. These results indicate that
3 NaRBOHD-based defenses play an important role in late herbivore-elicited responses (Wu *et al.*,
4 2013).

5 The evidence of ROS involvement in plant-insect interactions is growing. The accumulation of
6 reactive oxygen species seems to play a major role in determining the extent of tissue alterations
7 during gall morphogenesis (Carneiro *et al.*, 2014). The detection of enzymes related to ROS
8 production has been described for galls induced by sucking insects (de Oliveira and dos Santos
9 Isaias, 2010). The potential role of ROS in the defense systems of wheat (*Triticum aestivum*) and
10 rice (*Oryza sativa*) against Hessian fly (*Mayetiola destructor*) larvae reveals a rapid and
11 prolonged accumulation of H₂O₂ in wheat plants at the attack site during incompatible
12 interactions. Increased accumulation of both H₂O₂ and O₂^{•-} was detected in rice plants during
13 non-host interactions with the larvae. Class III peroxidases might play a role in ROS generation
14 in resistant wheat and non-host rice plants during the response to Hessian fly attacks (Liu *et al.*,
15 2010). Moreover, insect infestation impacts the stress signaling network through the effects on
16 the ROS and cellular redox metabolism, with particular emphasis on the roles of ROS in the
17 plant responses to phloem-feeding insects (Kerchev *et al.*, 2012). Pea aphid (*Acyrtosiphon*
18 *pisum*) causes oxidative stress conditions in pea leaves through the enhanced production of H₂O₂
19 and O₂^{•-}. The early, strong generation of H₂O₂ was observed at 24 h in aphid-infested leaves,
20 whereas the strong generation and continuous increase of O₂^{•-} production in aphid-infested
21 leaves from 0 to 96 h enhanced the defense potential to protect against aphid herbivory. The
22 relative release of H₂O₂ and O₂^{•-} was estimated with confocal microscopy by staining leaves
23 with specific fluorochromes [dichlorodihydro-fluorescein diacetate (DCFH-DA) and

1 dihydroethidium (DHE)] to reveal the fine-tuned regulatory mechanisms involved in plant-aphid
2 interactions (Van *et al.*, 2013).

3 The concentration of H₂O₂ and malondialdehyde (MDA) and the activities of several ROS
4 scavenging enzymes and related gene expression [e.g., superoxide dismutase (SOD), catalase
5 (CAT), and peroxidase (POD)] were measured in different species damaged by mechanical and
6 herbivore wounding. After infestation with chewing insects, the POD activity in the sap and total
7 soluble protein of tomato, cowpea and cotton were enhanced. Similar results were observed with
8 the feeding of sap sucking insects (Singh *et al.*, 2013). In poplar, the concentration of H₂O₂ and
9 MDA and the activities of SOD, CAT and POD were enhanced by herbivore wounding
10 treatment, but not as much as by mechanical wounding, suggesting that H₂O₂, SOD, CAT and
11 POD are associated with insect resistance (An *et al.*, 2010). In the “living fossil” plant *Ginkgo*
12 *biloba*, a significantly higher H₂O₂ production is observed 30 min after herbivory than in
13 mechanically damaged leaves; however, by 4 h after feeding, the values dropped to the control
14 levels. The use of DPI (diphenyleneiodonium, a suicide inhibitor of the phagocytic NADPH
15 oxidase and an inhibitor of NADH-dependent H₂O₂ production by peroxidase) inhibited
16 herbivory-dependent H₂O₂ production to the control levels, whereas verapamil (a voltage-gated
17 Ca²⁺ channel antagonist with a significant effect on herbivore-induced Ca⁺² release) significantly
18 increases the level of H₂O₂ upon herbivory. These data confirm the existence of an interplay
19 between H₂O₂ and calcium homeostasis (Mohanta *et al.*, 2012).

20 Another interesting question is how much insects can tolerate the increased ROS production in
21 the attacked leaves. In the emerald ash borer (*Agrilus planipennis*), antioxidant genes quench
22 ROS from both dietary and endogenous sources (Rajarapu *et al.*, 2011), whereas *Lymantria*
23 *dispar* caterpillar larvae can tolerate elevated levels of ROS in their midguts without nutritionally

1 significant changes in the compositions of susceptible essential amino acids in their food
2 (Barbehenn *et al.*, 2012). The abundance of vitamin C (VitC) in plants influences their
3 susceptibility to insect feeding as VitC is an essential dietary nutrient that serves as an
4 antioxidant in the insect midgut and is also a substrate for plant-derived ascorbate oxidase, which
5 can lead to the generation of toxic reactive oxygen species. Herbivores appear to influence both
6 the de novo synthesis and redox cycling of VitC in their host plants, thereby potentially altering
7 the nutritional value of crops and their susceptibility to pests. The recent development of
8 genetically modified crops with enhanced VitC content provides both an impetus and a tool for
9 further studies on the role of VitC in plant-insect interactions (Goggin *et al.*, 2010).

10 *Involvement of NRS*

11 The available descriptions of NO-mediated physiological processes are continuously increasing.
12 Although it was initially associated with plant defense responses against pathogens, reports
13 indicate that NO is also involved in plant responses to insects. *S*-nitrosoglutathione reductase
14 (GSNOR) reduces the NO adduct *S*-nitrosoglutathione (GSNO), an essential reservoir for NO
15 bioactivity involved in plant-herbivore interactions. Using a virus-induced gene silencing (VIGS)
16 system, the activity of GSNOR was knocked down in *N. attenuata*. The silencing of GSNOR
17 decreased the herbivory-induced accumulation of JA and ethylene without compromising the
18 activity of two mitogen-activated protein kinases (MAPKs), salicylic acid-induced protein kinase
19 (SIPK) and wound-induced protein kinase (WIPK) (Wuensche *et al.*, 2011a). *M. sexta* feeding
20 decreased the activity of trypsin proteinase inhibitors (TPIs), which were detected in GSNOR-
21 silenced plants. Therefore, GSNOR plays an important role in plant resistance to herbivory and
22 JA signaling and suggests the potential involvement of NO in plant-herbivore interactions
23 (Wuensche *et al.*, 2011a).

Nitric oxide-associated protein 1 (NOA1) is required for plant resistance in plant-herbivore interactions. NOA1-silenced *N. attenuata* plants treated with mechanical wounding and *M. sexta* OS showed elevated levels of herbivory-induced JA but reduced concentrations of most carbon-based defensive compounds. These data suggest the involvement of NOA1 in *N. attenuata*'s defense against *M. sexta* attack, and highlight its role in photosynthesis and in the biosynthesis of jasmonates and secondary metabolites (Wuensche *et al.*, 2011b).

Nilaparvata lugens, the brown plant hopper (BPH), is one of the most destructive phloem-feeding insects to attack rice throughout Asia. BPH feeding increases the level of endogenous NO in the leaf and sheath tissue of both resistant and susceptible rice cultivars within 1 h of infestation. The production of NO in response to BPH feeding appears to depend primarily on the activity of nitric oxide synthase (NOS). The application of exogenous NO stimulated the expression of certain drought stress-related genes, reduced the plant height, delayed leaf senescence and reduced the seedling mortality caused by BPH feeding. This suggests that NO signaling plays a role in the rice tolerance response to BPH feeding (Liu *et al.*, 2011). In the interaction between *S. littoralis* and Lima bean, both herbivory and robotic wounding induced marked accumulation of NO but with distinct temporal patterns. Herbivory caused a rapid and transient increase of NO levels, whereas the response to robotic wounding was of equal intensity but delayed (Bricchi *et al.*, 2010). Interestingly, the time course of NO production parallels the induction of the volatile compound emission observed in Lima beans (Arimura *et al.*, 2011), suggesting a possible involvement of NO in the signaling pathway leading to volatile emission. Figure 2 summarizes the changes in the ROS and RNS signaling pathways in response to herbivory.

A rapid chemical signal provides a systemic warning

1 Systemic responses are used by plants to prevent attacking herbivores from moving from
2 wounded zones, where plant defenses are built up in response to feeding, to reach unattacked
3 leaves. The existence of such a systemic signaling molecule was first reported in tomato plants in
4 the pioneer studies of Ryan and co-workers (Green and Ryan, 1972). Since then, regulation of
5 defense genes in systemic tissues upon herbivory has been increasingly reported (Champigny
6 and Cameron, 2009), and considerable research effort has focused on the identification of
7 systemic wound signals and the mechanisms by which they are generated, transported and
8 perceived in distal leaves. The signaling species implicated in wound-induced systemic
9 responses include oligosaccharides, reactive oxygen species, systemin, green leaf volatiles,
10 hydraulic signals, electrical signals and various plant hormones (Koo *et al.*, 2009 and references
11 cited therein). The nature of the systemic wound signal remains unknown. In the current model, a
12 rapidly transmitted wound signal triggers the systemic synthesis of JA, which, upon conversion
13 to JA-Ile, activates the expression of early response genes by the SCF^{COI1}/JAZ pathway (Koo *et*
14 *al.*, 2009). In addition to the responses in the attacked leaf, the enhanced levels of defense gene
15 transcripts are found in unelicited leaves on the same plant. This between-leaf systemic signaling
16 of defense responses occurs without MAPK activation in systemic leaves and suggests the
17 existence of a mobile signal other than the one activating responses within the attacked leaves
18 (Wu *et al.*, 2007). However, the nature of the signal and its means of propagation are still
19 debated. Given how rapidly MAPKs are activated in undamaged regions, it may be that the
20 signal is carried by electricity or rapidly propagated hydraulic signals. Another hypothesis is that
21 relatively small molecules, such as FACs or FAC-elicited signal compounds, enter the xylem
22 vessels through wounds and are carried to specific regions of the leaf (Wu *et al.*, 2007). Whether
23 electric signaling or xylem transport is involved in activating MAPKs in undamaged regions

1 needs further investigation. Increasing evidence excludes the possibility that plant
2 phytohormones (e.g., JA or its derivatives such as the JA isoleucine conjugate or its methyl ester)
3 might be the travelling molecules because they are synthesized in the systemic leaves rather than
4 transported (Wang *et al.*, 2008); moreover, insect elicitors induce a higher JA accumulation in
5 distant tissues (VanDoorn *et al.*, 2010; Bricchi *et al.*, 2013).

6 Quite recently, evidence on the role of the symplastic connection for the transmission of the
7 moving signal was provided. Hydroponically cultured *Arabidopsis* provides an ideal tool to
8 study shoot-to-root signaling, allowing for changes in V_m in the root to be measured in real time
9 following biotic or chemical stress on the shoots. When the root- V_m was measured in WT
10 *Arabidopsis*, it was found to be significantly depolarized 7 - 8 min after either herbivory or the
11 application of OS or FR α , a β -galactofuranose isolated from *S. littoralis* OS. On the other hand,
12 the *pdko3* mutant showed no root- V_m depolarization after herbivory or the application of either
13 OS or FR α . The rate of signal transduction was $\sim 110 \text{ cm h}^{-1}$, which matches the rate of phloem
14 transport (from 30 to 150 cm h^{-1}). To test the phloem transport hypothesis, petioles of the treated
15 leaves were subjected to either cold (4°C) or heat (60°). Both treatments completely abolished
16 the WT herbivore-, OS- and FR α -induced root V_m depolarization, indicating the requirement of
17 functional sieve elements. Therefore, because the *pdko3* mutant does not transmit the signal, it
18 appears that that disruption of plasmodesmata function reduces the systemic signaling (Bricchi *et*
19 *al.*, 2013). From all these studies, it is clear that elucidating the nature of the mobile signals in
20 different species will contribute to an understanding of the rates of signal transmission and the
21 speed of systemic plant responses to herbivory. In our laboratory, we tested different species and
22 analyzed the time of root V_m depolarization upon leaf herbivory (*S. littoralis*). The rate of
23 transmission was found to correspond to the phloem rate (Maffei *et al.*, unpublished results).

1 However, different biotic attacks may trigger different rates of plant responses. For instance, in
2 *Arabidopsis*, the plasma membrane responds with a Vm depolarization depending on the nature
3 of biotic attack. The Vm response is much more rapid upon *S. littoralis* (30 min -2 h) herbivory
4 than following an attack by the aphid *Myzus persicae* (4-6 h). Furthermore, pathogenic infection
5 with *Pseudomonas syringae* induces a very late Vm depolarization (16 h). Regardless of the
6 nature of the biotroph and the rate of response, the Vm depolarization occurs at the same
7 intensity, which in *Arabidopsis* corresponds to a Vm depolarization of approximately 40 mV.
8 This suggests the occurrence of a Vm threshold, which is reached for successful
9 herbivory/infection (Bricchi *et al.*, 2012). The question of why the Vm polarization occurs at
10 different times appears to be associated with the mode of the biotic damage. The fast clipping
11 and consistent plant tissue removal by chewing herbivores induces a "quantitative" response that
12 is proportional to the level of tissue damage; on the other hand, the stylet probing and the phloem
13 feeding of aphids induces a reduced level of damage that requires more time for a plant response.
14 Finally, bacterial growth and tissue damage takes time, which appears to be proportional to the
15 Vm depolarization. Therefore, besides the nature of the moving molecule, it will be important to
16 evaluate the nature of the elicitor that induces its synthesis.

17 Because the use of plasmodesmata mutants in *Arabidopsis* suggests the involvement of phloem
18 elements as the possible carriers of the moving signal(s), this hypothesis restricts the molecular
19 size of the moving signal to plasmodesmata constraints, including the flux rate [$3.3\text{--}8.5\ \mu\text{m s}^{-1}$,
20 (Rutschow *et al.*, 2011)], their specificity and directionality (Christensen *et al.*, 2009), their
21 variable flux properties between species and tissue types (Maule *et al.*, 2011), the role of ROS as
22 potent molecules that have an impact on the efficiency of PD trafficking (itez-Alfonso *et al.*,
23 2011) and their size exclusion limits (Burch-Smith and Zambryski, 2012; Lee, 2014). Figure 3

illustrates a symplastic model for the transmission of the moving signal as well as for the effects of disrupting the symplastic continuity of the systemic response.

Concluding remarks

Depolarization of the V_m is one of the first responses of the plasma membrane. It depends mainly on the ion fluxes, including the opening of K^+ channels, calcium release from internal stores or influx from the apoplast. The cytosolic calcium increase triggers protein calcium sensors including calmodulin, calcium-dependent protein kinases and calcineurin signaling, which activate a cascade of signals and eventually leads to transcription factor activation and transcriptional regulation. Calcium also triggers hydrogen peroxide production through the activation of CBL1/CBL9-CIPK26 interaction, which eventually phosphorylates RBOH and thus activates the production of ROS. Calcium homeostasis changes and cell redox status alterations also trigger NO production, which can either directly or indirectly affect the plant cell responses to herbivory. A growing body of evidence indicates the presence of mobile signaling molecules that travel from the wounded tissues towards systemic organs. Although the nature of this molecule remains unknown, its presence depends on the activity of insect's oral secretions, where specific elicitors (FACs or oligosaccharides) are necessary and sufficient to start the response process. Both the xylem and phloem are good candidates, given the path of the molecule toward the systemic tissues. In *Arabidopsis*, the elicitation with *S. littoralis* β -galactofuranose requires functioning plasmodesmata in order for the signaling molecule to reach the systemic tissues, justifying a model where the symplastic route is used.

Understanding fast responses of plants to the surrounding environment is of interest not only from an ecological and evolutionary perspective but also for the development of novel crop

1 protection strategies. Owing to the massive damage that herbivores cause to valuable crops, the
2 deciphering of early signals from plants represents one of the most exciting areas research in the
3 first line of defense.

4 Three areas where future efforts might result in major breakthroughs are related to the
5 identification of herbivore-specific signal molecules, their recognition, and further signal
6 transduction. The challenge for further research is to determine their mode of action, whether
7 these signals are transduced by receptor-mediated processes or whether they simply interact with
8 the plant membranes to initiate signal transduction pathways. One approach to achieve this goal
9 is the use of plant mutants that are not responsive to a particular herbivory-related signal, as the
10 *Arabidopsis pdko3* mutant or the several *N. attenuata* silenced plants (Yang *et al.*, 2012; Bricchi
11 *et al.*, 2013; Woldemariam *et al.*, 2013; Wu *et al.*, 2013). The characterization and use of such
12 mutants could result in the identification of genes encoding proteins involved in signal
13 perception. Such studies can not only uncover individual signaling pathways but can also
14 establish links in a network of alternative routes regulating the multitude of inducible plant
15 defenses.

Accepted Article

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Figure 1. Calcium signaling pathways after herbivory. Following herbivory, insect-originated elicitors bind on putative polysaccharide (β -galactofuranose) or FACs receptors, leading to cytosolic Ca^{2+} homeostasis. This homeostasis is regulated by influx and efflux channels as well as by ATP-dependent Ca^{2+} pumps. The cytosolic Ca^{2+} increase induces the release of Ca^{2+} from cellular stores (mitochondria, vacuoles and the endoplasmic reticulum) via Ca^{2+} channels. The cytosolic Ca^{2+} increase triggers two cascades of signaling events: 1) the activation of inward K^+ channels, which cause V_m depolarization, and 2) the activation of CBL-interacting protein kinases (CBL-CIPK), calmodulin-like protein (CML42 & CML43), and calcium dependent protein kinases (CPK3 & CPK13) signaling pathways, which are involved in the activation of transcription factors (e.g., HSF2A) that lead to the expression of defense genes in the nucleus. The broken arrows indicate the calcium pathways contributing to the increased cytosolic calcium concentrations.

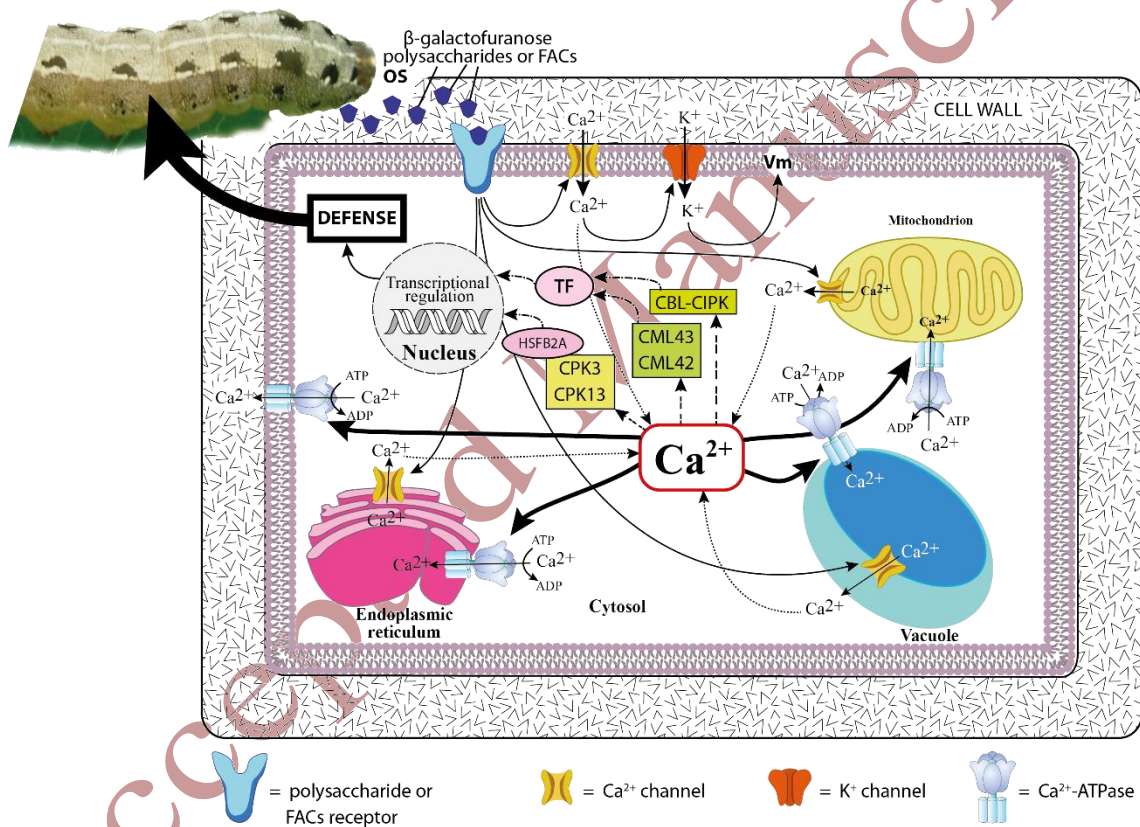


Figure 2. The role of ROS and NRS in plant-insect interactions. Upon herbivory, insect originated elicitors bind putative polysaccharide (β -galactofuranose) or FACs receptors, leading to the cytosolic Ca^{2+} homeostasis (see Figure 1). The cytosolic Ca^{2+} increase triggers the CBL-CIPK signaling pathways, which activate the plasma membrane NADP oxidase (ROBH). This in turn generates the superoxide radical anion, which is eventually dismutated to hydrogen peroxide (H_2O_2) by the catalytic activity of superoxide dismutase (SOD). The activity of ROBH is also regulated by external ATP. H_2O_2 acts directly on herbivores or enters the cytosol through hydroperoxiporins, increasing the cytosolic H_2O_2 concentration. By the action of mitochondrial Fenton reaction and the activity of catalase (CAT) peroxidase (POD) and ascorbate peroxidase (APX), the levels of H_2O_2 are reduced. In contrast, the activity of peroxisomal xanthine oxidase, mitochondrial CIP1/CIP3, Ubiquinone, and chloroplastic PSI catalyze the conversion of O_2 to superoxide radical anion ($\text{O}_2^{\bullet-}$), which contributes to the elevation of the H_2O_2 cytosolic concentration by the action of SOD. Cytosolic Ca^{2+} increase may lead to nitric oxide synthase (NOS) activity, thus increasing the cytosolic NO level. The NO adduct *S*-nitrosoglutathione (GSNO) level is reduced by the activity of *S*-nitrosoglutathione reductase (GSNOR), producing the oxidized glutathione disulphide (GSSG) and NH_3 . Nitric oxide-associated protein 1 (NOA1) and *N*-nitroso species, as well as the activity of GSNOR have been associated with the phytohormone JA, triggering the transcriptional regulation of defense genes.

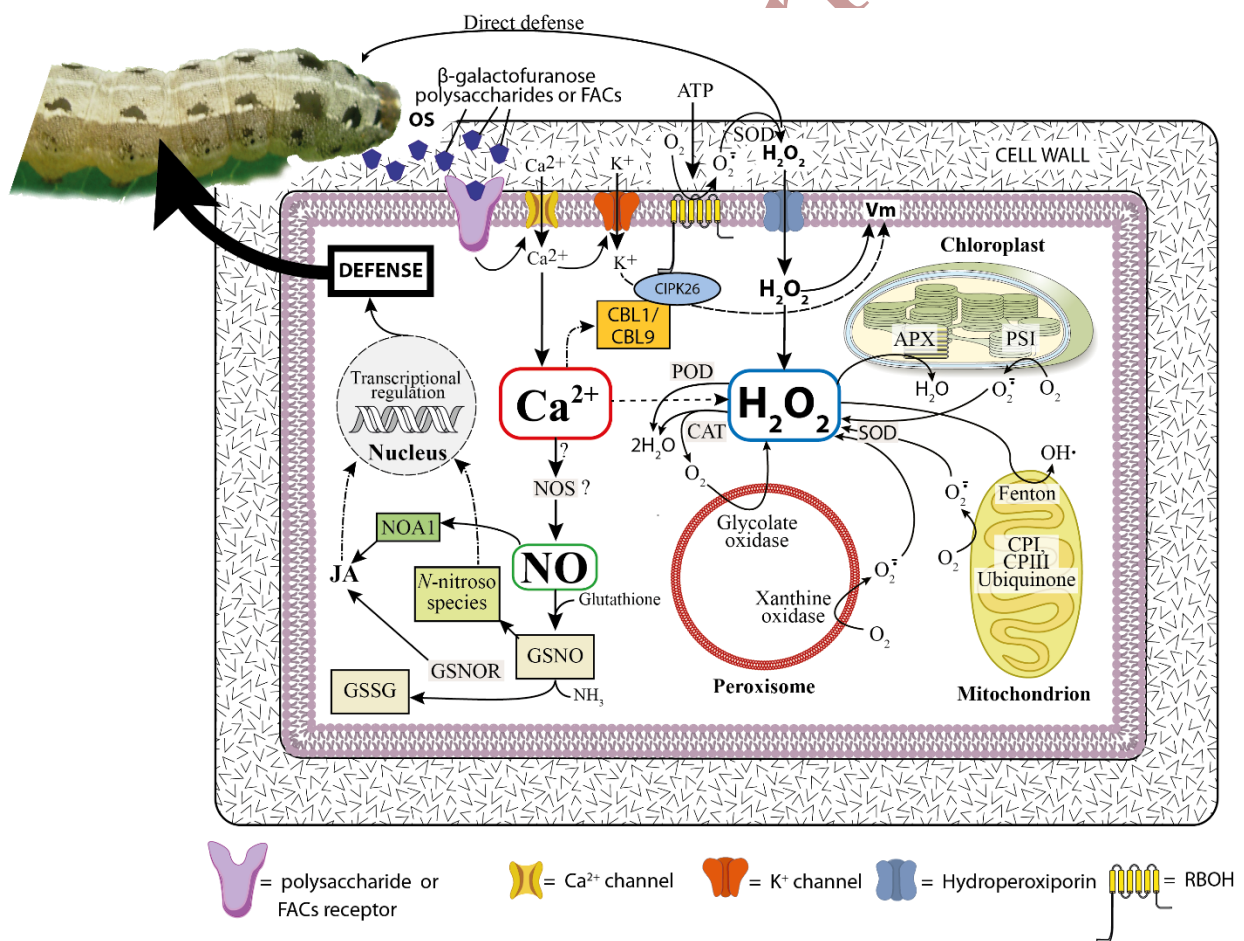


Figure 3. *Arabidopsis* model for the transmission of a moving signal from wounded to systemic tissues. Upon herbivory and the release of factors present in insect oral secretions (including a putative β -galactofuranose polysaccharide and/or FAC), both the *pdko3* and wild type (WT) plants show an increased accumulation of $[Ca^{2+}]_{\text{cyt}}$, NO and H_2O_2 . In contrast, unlike WT plants, the mutant shows an almost complete loss of voltage-gated K^+ channel activity and Vm depolarization, a loss of shoot-induced root-Vm depolarization, a loss of gene expression activation and regulation of the JA defense pathway and a much diminished release and altered profile of the VOCs. These events implicate the symplastic route as probable candidate for the means by which moving molecules reach systemic tissues.

